Docket No. 4010.3002US2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Mark A. Holland and Nicole Lenihan

Application No: 10/821,640 Group Art Unit: 1648

Filed: April 9, 2004 Examiner: Nicole Kinsey

Confirmation No.: 9018

Title: BACTERIOPHAGE FOR LYSIS OF METHYLOBACTERIUM AND COMPOSITIONS AND USES THEREOF

DECLARATION UNDER §1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Richard M. Carlton, M.D., am the president of CST Technology Group, LLC 3 Secor Drive Port Washington, NY 11050, the exclusive licensee of the above-identified application. I hereby declare that:

I have reviewed the outstanding Office Action dated October 9, 2007 and the patent application. I understand that in examining the above-identified patent application, a concern has been raised as to whether the phage preparation that Professor Holland submitted to the repository is a unique, one-of-a-kind phenomenon, or, instead, if it represents something that is generalizable (repeatable). There is abundant evidence, from a variety of authoritative sources (reviewed below), that makes it clear that this is in fact something generalizable. Phages are abundant and ubiquitous in the environment, and are

found wherever bacteria are found. Moreover, Professor Holland did in fact isolate multiple different strains of anti-PPFM phages.

One of the authoritative textbooks available on the general subject is a two-volume text entitled Viruses of Prokaryotes, edited by H.-A. Ackermann and M. Dubow, CRC Press, 1987. The authors point out that "Phages have the same habitats as their hosts", meaning of course that wherever the hosts (bacteria) are found, phages that target them will also be found. In Volume 1 of that textbook, in Chapter 3 (which is entitled "Occurrence and Frequency of Bacteriophages"), on page 37 one will find Table 4, Habitats of Bacteriophages. (Exhibit A.) This table documents that the known phage habitats include water, soil, air, plants, animals, and food. I will focus here on the phages found in soil and on plants:

- Soil
  - Types of soil: arable, clay, compost, forest, moor, mud and silt, podzol, rhizosphere, sand
  - From: alfalfa, soybean, and vegetable fields; barnyards, cow sheds, chicken houses, gardens, greenhouses, lawns.
- Plants:
  - Organs: buds, leaves, nodules (leguminous plants), rotting fruits, roots, seeds, stems and straw; crown gall tumors
  - Species: healthy or diseased alfalfa, barley, beans, broccoli, Brussels sprouts, buckwheat, clover, cotton, cucumber, oats, peas, peach trees, radish, rutabaga, rye, timothy, tobacco, tomatoes, wheat.

Wikipedia has an informative site dedicated to phage ecology, with the following link: http://en.wikipedia.org/wiki/Phage_ecology#Vastness_of_phage_ecology The site says: "As a rule of thumb, many phage biologists expect that phage population densities will exceed bacterial densities by a ratio of 10-to-1 or more (VBR or virus-to-bacterium ratio; see [1] for a summary of actual data). As there exist estimates of bacterial numbers on Earth of approximately 10^{30}[2], there consequently is an expectation that 10^{31} or more individual virus (mostly phage[3]) particles exist[4], making phages the most numerous category of "organisms" on our planet." (Exhibit C).

Reference number [3], which was cited in that quotation from the above web site, is the following article: Wommack, K. and Colwell, R. Viroplankton: Viruses in Aquatic Ecosystems. *Microbiol Mol Biol Rev.* 2000 March; 64(1): 69–114. (Exhibit D). This article confirms that when counting the numbers of bacteria and phages in ocean waters, phages are found at "an order of magnitude more frequency" than bacteria. Importantly, one of the co-authors of that article, Dr. Rita Colwell, is a past President of the American Society of Microbiology, and a great authority on such matters.

With the above background in mind, we can now explain the reason that Professor Holland was motivated to find a phage that would lyse PPFMs:

Professor Holland had been progressing nicely in a series of experiments designed to demonstrate that PPFM bacteria are essential for plant growth and function, largely because they secrete two hormones (cytokinin and auxin) that are well known in the literature to be crucial "plant" hormones. But prior to his experiments, no one realized that the PPFMs gave meaningful levels of those hormones to plants they were colonizing, and that this "gift" makes a big difference.

In order to prove that the PPFMs and their hormones make a difference, he had been using heat to "cure" seeds of their endogenous supply of PPFMs. He would heating them to a temperature (25°C) and duration (24 hr) sufficient to knock back the population of the bacteria, while hoping to keep the damage to the seed's germ as minimal as possible. These experiments showed that the "cured" seeds failed to germinate; but that when those cured seeds were soaked in a solution of (exogenous) PPFMs, germination was restored.

Professor Holland then set out to find a way to cure seeds of their endogenous PPFMs that would be *less disruptive than heating*, because the heat was definitely stunting the plants to a certain extent. It occurred to him that using a phage targeting Methylobacterium species (such as Methylobacterium mesophilicum) would serve that purpose well.

When he checked the public phage collections (e.g. the collection held by American Type Culture Collection), there were no phages listed against PPFMs. A search of some private phage collections also turned up negative. He therefore set out to isolate and propagate an anti-PPFM phage on his own. To do so he collected samples from the floor drains and around the drainage of flower pots in the greenhouse on the college campus. In short time, he was successful in isolating a number of different phage strains that were virulent for PPFMs (that is, that clearly and unambiguously lysed the target bacteria). He was able to discern that there were different strains (as opposed to multiple copies of the same strain) because the plaques that they produced, on a lawn of the Methylobacterium host, had different sizes and appearances. Out of the many different phage strains that were isolated, he selected one at random that was particularly virulent (making the clearest plaque, with no "turbidity" within the plaque which would indicate that there were some bacterial survivors that were growing), and he submitted that strain to the repository.

When he used this phage in an attempt to cure seeds of their endogenous strains of PPFMs, it worked quite well: Germination was stunted. And adding exogenous PPFMs helped to restore germination. As an important aside, the reason that the phages did not stop germination completely is that seeds have PPFM bacteria in their interior as well as on the seed coat, and naturally the phages would not be able to access and lyse the target bacteria in the interior. Please see Figure 1 below.

Figure 1. Phage-cured seeds germinate poorly

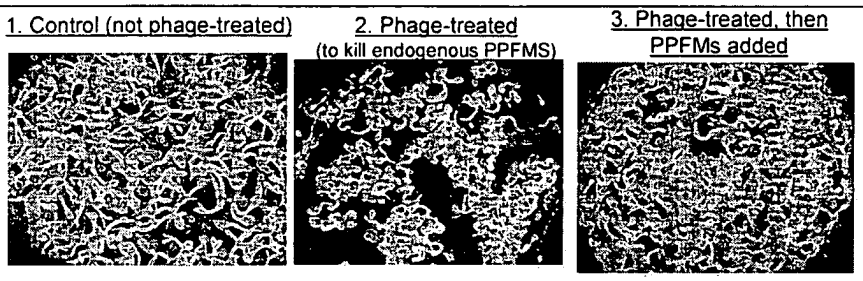


Fig. 1. Arabidopsis seeds are allowed to germinate. In this Figure, the control batch, germination takes place under normal circumstances

Fig. 2. The experimental batch in the middle has been incubated with anti-PPFM bacteriophages, which have killed most of the PPFM bacteria

- Fewer control seeds have sprouted, and the sprouts are much smaller
- The control sprouts have less chlorophyll

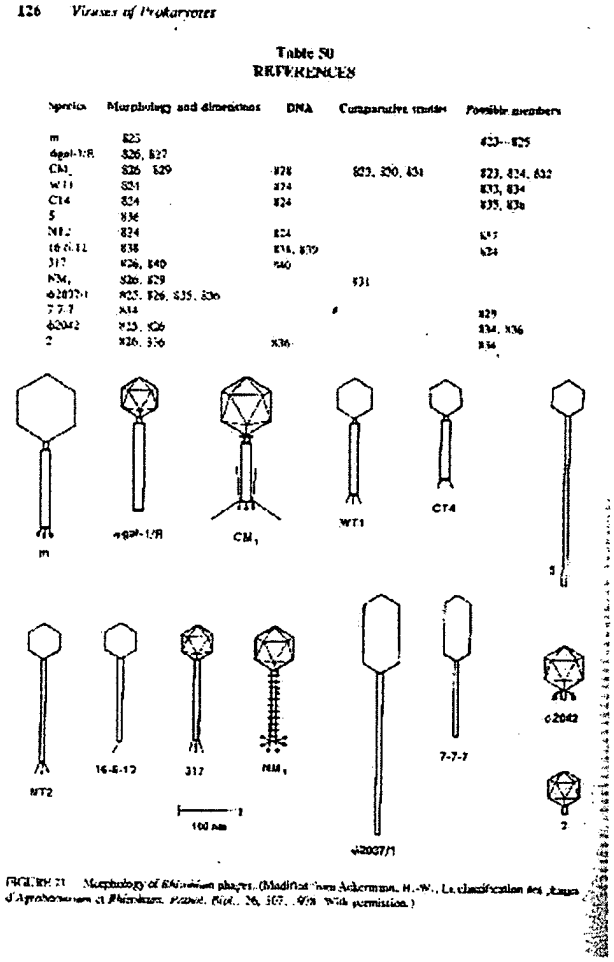
Figure 3. Samples of those same phage-treated seeds were then supplemented with exogenous PPFM bacteria. Notice how germination has been restored, essentially to normal. These experiments show that PPFMs are essential for the proper germination of seeds.

Once Professor Holland had obtained that virulent anti-PPFM phage strain, and had demonstrated that it worked well in decreasing the rate (and vigor) of germination of seeds, he had no scientific need to search and isolate even more such phage strains, in addition to the variety he had already obtained. His quest was not that of a phage epidemiologist, who would want to find out how many strains of phages (against the target bacterial species) are present, or what they look like morphologically, or what their genetics are.

However, had he wanted to find a wide variety of phage strains that target PPFMs, the collective experience of thousands of phage researchers around the world would predict that he would in fact have found a smorgasbord of different phage strains. To illustrate this point, please see Figure 2 below, which is a scan of p.126 from Volume II of Viruses of Prokaryotes, which lists (and shows the morphology of) a sampling of the phages that

have been isolated against Rhizobium – a bacterial species that is fairly closely related to the Methylobacteria.

Figure 2. There are numerous phages of Rhizobia (a species close to the Methylobacteria)



As explained by the authors of that textbook, scientists had good reasons to look for a variety of different phage species and strains that target the Rhizobia bacteria: "Rhizobia bacteria are extremely valuable for agriculture (for their nitrogen-fixing activities) and

are used to inoculate seeds before planting. One of the goals of genetic engineering is to confer their nitrogen-fixing ability to other bacteria or to plants". Naturally, scientists would turn to phages to assist in that genetic engineering; but they would need to find phages that infect this particular genus of bacteria, the Rhizobia. The authors continue "Phages have been found in *R. japonicum, R. leguminosarum, R. lupine, R. meliloti, R. phaseoli, R. trifolii*, and less well-defined organisms, i.e., Galega or lotus Rhizobia.

In contrast to the situation for the Rhizobia, there has not been a corresponding interest (either scientific or commercial) to find phages against the PPFMs. This helps explain why none were available to Professor Holland when he set out to obtain one for his "seed-curing" experiment, and therefore had to find one on his own.

As someone knowledgeable in the field of phages and their practical applications for mankind, it is my opinion that if one were to make the effort to find additional phage strains against PPFMs, using the guidance provided in the specification, numerous such phages would be found absent undue experimentation.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Sincerely,

Richard M. Carlton, M.D.

Richard M. Carlton, M.D.
President